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ARTICLENorepinephrine and its metabolites are involved in
the synthesis of neuromelanin derived from the
locus coeruleus

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Abstract

In order to elucidate the chemical structure of black to brown pigments, neuromelanins (NMs), in the substantia nigra (SN) and the *locus coeruleus* (LC) in the central nervous system of humans and other mammalian species during aging, chemical degradative methods are powerful tools. HPLC analysis after hydroiodic acid hydrolysis detected aminohydroxyphenylethylamines, aminohydroxyphenylacetic acids, and aminohydroxyethylbenzenes, which confirmed that SN-NM and LC-NM contain melanin derived not only from dopamine and norepinephrine (NE) but also from several other catecholic metabolites, such as 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylethanol, and 3,4-dihydroxyphenylethylene glycol,

in addition to the corresponding Cys-derivatives in varying degrees. However, hydroiodic acid hydrolysis showed that LC-NM produced the same degradation products as were detected in SN-NM. Thus, we needed to develop a new chemical detection method to validate the existence of NE in LC-NM. In the present study, we report that HCl hydrolysis of LC-NM in the presence of thioglycolic acid yields new products arising from substitution of the hydroxyl group by thioglycolic acid at the benzyl position of NE and cysteinyl-NE. This is the first chemical evidence showing that NE and cysteinyl-NE are incorporated into LC-NM.

Keywords: HCl hydrolysis, *locus coeruleus*, neuromelanin, Parkinson's disease, substantia nigra.

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Neuromelanins (NMs) are complex polymeric compounds present in the human central nervous system that are localized in cytoplasmic organelles surrounded by a double membrane, together with lipid bodies and proteins (Sulzer *et al.* 2008; Zecca *et al.* 2008). NM pigment itself is composed of different compounds: melanin, proteins, lipids, and metal ions (Zecca *et al.* 2008; Engelen *et al.* 2012; Wakamatsu *et al.* 2012). NM is synthesized mainly in dopaminergic neurons of the substantia nigra (SN) and in noradrenergic neurons of the *locus coeruleus* (LC), however, it has been demonstrated that NM is also synthesized and accumulates in neurons of other brain areas (Zecca *et al.* 2008). The synthesis and accumulation of NM inside neurons arise during brain aging and there is evidence that NM is involved in the pathogenesis of neurodegenerative diseases such as Parkinson's disease (PD) (Mann and Yates 1983;

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Abbreviations used: 5-S-Cys-CMT-DA, 5-S-cysteinyl β-(carboxymethylthio)dopamine; 5-S-Cys-DA, 5-S-cysteinyl-dopamine; 5-S-Cys-NE, 5-S-cysteinyl-norepinephrine; AHEB, aminohydroxyethylbenzene; AHPAA, aminohydroxyphenylacetic acid; AHP, aminohydroxyphenylalanine; AHPEA, aminohydroxyphenylethylamine; CMT-DA, β-(carboxymethylthio)-dopamine; Cys, cysteine; DA, dopamine; DOMA, 3,4-dihydroxymandelic acid; DOPA, 3,4-dihydroxyphenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPE, 3,4-dihydroxyphenylethanol; DOPEG, 3,4-dihydroxyphenylethylene glycol; HI, hydroiodic acid; LC, *locus coeruleus*; LC-NM, NM from LC tissue; NE, norepinephrine; NM, neuromelanin; PDCA, pyrrole-2,3-dicarboxylic acid; PD, Parkinson's disease; PTCA, pyrrole-2,3,5-tricarboxylic acid; SN-NM, NM from SN tissue; SN, substantia nigra; TDCA, thiazole-4,5-dicarboxylic acid; TTCA, thiazole-2,4,5-tricarboxylic acid.

Marsden 1983; Zecca *et al.* 2006). Notably, the SN and LC are the brain areas with the highest concentration of NM pigment in the brain (Zecca *et al.* 2004).

In contrast to cutaneous melanin, the biosynthesis and structure of NM remain poorly understood, especially NM derived from the LC (LC-NM) due to the paucity of material and the difficulty of its isolation. In fact, the majority of structural studies on NMs have employed NM isolated from the SN (SN-NM) and other large areas of the human brain (Wakamatsu *et al.* 2003, 2012; Zecca *et al.* 2008).

We have previously performed chemical analyses in order to elucidate the structure of NM in the SN (Wakamatsu *et al.* 2003, 2012; Zecca *et al.* 2008). In addition, it was recently suggested that various catecholic metabolites are incorporated into NM, including 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxymandelic acid (DOMA), 3,4-dihydroxyphenylethanol (DOPE) and 3,4-dihydroxyphenylethylene glycol (DOPEG), which are metabolites of DA and norepinephrine (NE) formed by the oxidative deamination by monoamine oxidase followed by oxidation/reduction (Fig. 1) (Wakamatsu *et al.* 2014).

To estimate the incorporation of various melanin precursors into NM, we used the chemical degradation approach of SN-NM originally adapted for the analysis of cutaneous melanin (Ito and Fujita 1985; Ito and Wakamatsu 2003). The reductive hydroiodic acid (HI) hydrolysis of SN-NM and LC-NM from the human brainstem followed by HPLC analysis using newly synthesized *o*-aminophenol compounds as standards (Fig. 2), i.e., aminohydroxyphenylethylamine isomers (AHPEAs), aminohydroxyphenylacetic acid isomers (AHPAAs) and aminohydroxyethylbenzene isomers (AHEBs), made it possible to confirm that SN-NM and LC-NM contain melanin derived not only from DA and NE

but also from several other catecholic metabolites, such as DOPA, DOPAC, DOMA, DOPE, and DOPEG, in addition to the corresponding Cys-derivatives in varying degrees (Wakamatsu *et al.* 2014). Those results indicated that most of the catechol derivatives noted above were incorporated into SN-NM and LC-NM during their biosynthesis. However, upon HI hydrolysis, LC-NM produced the same degradation products, AHPEAs, AHPAAs, and AHEBs, as SN-NM did (Wakamatsu *et al.* 2014). This is because the hydroxyl group at the benzyl position of NE (and its metabolites) is easily eliminated to produce DA (and its metabolites) under the conditions of reductive HI hydrolysis. Thus, we could not distinguish LC-NM from SN-NM by HI reductive hydrolysis.

Thus, as far as we know, there have been no reports regarding whether NE is incorporated into NM, and to what extent NE and NE metabolites are present, especially in LC-NM, which accumulates in LC neurons that contain high levels of NE. Thus, we developed a new chemical detection method to validate the existence of NE in LC-NM, and the contents of NE metabolites in LC-NM. We report here the first chemical evidence regarding the existence of NE and NE metabolites in LC-NM.

Materials and methods

DA, NE, Cys, DOMA, DOPEG, mushroom tyrosinase (EC.1.14.18.1, 1,715 units/mg), and 57% HI were purchased from Sigma-Aldrich (St Louis, MO, USA). DOPE was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 50% H₂O₂, 30% H₂O₂ and sodium 1-octanesulfonate were purchased from Nacalai Tesque, Inc (Kyoto, Japan). DOPAC and Dowex 50W-X2 (200–400 mesh) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Preparations of pyrrole-2,3,5-tricarboxylic acid (PTCA), pyrrole-2,3-dicarboxylic acid (PDCA), thiazole-2,4,5-tri-

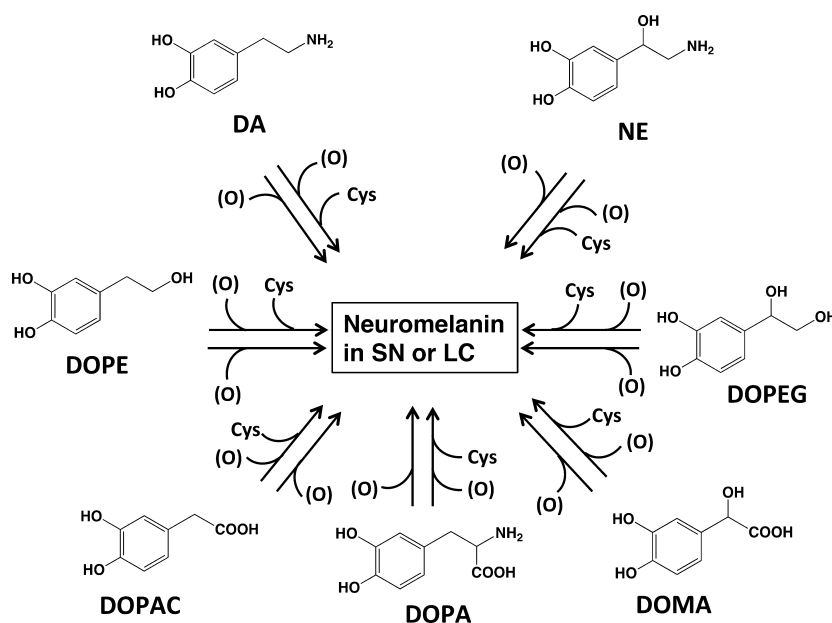


Fig. 1 Possible participation of various catecholic metabolites known to be present in various regions of the brain that may be incorporated into neuromelanin (NM). In addition to dopamine (DA) and norepinephrine (NE) and the corresponding Cys-derivatives, these other metabolites are also thought to be incorporated into NM. (O) represents the oxidants.

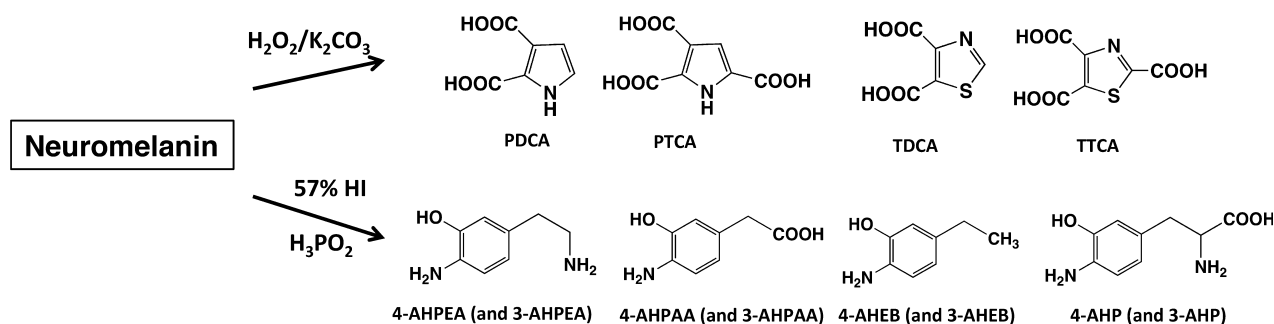


Fig. 2 Various melanin markers of neuromelanin (NM) produced by chemical degradative methods. On alkaline H₂O₂-mediated oxidation, melanins derived from 3,4-dihydroxyphenylalanine (DOPA), dopamine (DA), norepinephrine (NE), and the corresponding Cys-derivatives gave pyrrole-2,3-dicarboxylic acid (PDCA), pyrrole-2,3,5-tricarboxylic acid (PTCA), thiazole-4,5-dicarboxylic acid (TDCA) and thiazole-2,4,5-tricarboxylic acid (TTCA), while melanins from Cys-derivatives of 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxymandelic acid

carboxylic acid (TTCA), thiazole-4,5-dicarboxylic acid (TDCA), 4-amino-3-hydroxyphenylalanine (4-AHP), 3-amino-4-hydroxyphenylalanine (3-AHP), 4-amino-3-hydroxyphenylethylamine (4-AHPEA) and 3-amino-4-hydroxyphenylethylamine (3-AHPEA) were described previously (d'Ischia *et al.* 2013). 5-*S*-cysteinyldopamine (5-*S*-Cys-DA) and 5-*S*-cysteinylnorepinephrine (5-*S*-Cys-NE) were prepared as described by Ito *et al.* (1986). 4-amino-3-hydroxyphenylacetic acid (4-AHPAA), 3-amino-4-hydroxyphenylacetic acid (3-AHPAA), 4-amino-3-hydroxyethylbenzene (4-AHEB) and 3-amino-4-hydroxyethylbenzene (3-AHEB) were prepared according to Wakamatsu *et al.* (2014). Pheomelanins derived from the Cys-derivatives of DA, NE, DOPAC, DOMA, DOPEG, DOPE, and DOPA, were prepared according to methods previously reported by our laboratory (Ito *et al.* 1986; Ito 1989). Alkaline H₂O₂-mediated oxidation and reductive HI hydrolysis were performed as described in Ito *et al.* (2011), and Wakamatsu *et al.* (2002), respectively. All other chemicals were of the highest purity commercially available. UV-visible spectra were recorded with a JASCO V-630 UV/VIS spectrophotometer (JASCO Co., Tokyo, Japan). The HPLC systems consisted of a JASCO 880-PU pump, a JASCO C₁₈ column (JASCO Catecholpak; 4.6 × 150 mm; 7 μm particle size) with a Shiseido 3005 SI-2 (Shiseido, Tokyo, Japan) electrochemical detector or a Shiseido C₁₈ column (Shiseido Capcell Pak MG; 4.6 × 250 mm; 5 μm particle size) with a JASCO UV detector (JASCO Co.) and a flow rate of 0.7 mL/min. ¹H-NMR (400 MHz) and ¹³C-NMR (100 MHz) spectra were recorded on a Bruker AVANCE400 spectrometer (Bruker, Billerica, MA, USA) and are reported as the chemical shift δ (ppm) downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate used as an internal reference. Coupling constants (*J*) are expressed in Hz and signals are expressed as s (singlet), d (doublet), or t (triplet). Elemental analyses were carried out by the Organic Elemental Analysis Research Center (Kyoto University, Japan). Mass spectra were obtained with an Applied Biosystems, a Biospectrometry Workstation Mariner (mode: electrospray ionization – time of flight, positive; ESI (+)-TOF) (Applied Biosystems, Foster City, CA, USA), and ion trap liquid chromatography/mass spectrometry was carried out using an Esquire HCT Plus Ion Trap Mass Spectrometer (Bruker) connected

(DOMA), 3,4-dihydroxyphenylethanol (DOPE) and 3,4-dihydroxyphenylethylene glycol (DOPEG) gave only TDCA and TTCA. On hydroiodic acid (HI) hydrolysis, melanins derived from the Cys-derivatives of DA (and NE), DOPAC (and DOMA), DOPE (and DOPEG) and DOPA gave aminohydroxyphenylethylamine (AHPEA) isomers, aminohydroxyphenylacetic acid (AHPAA) isomers, aminohydroxyethylbenzene (AHEB) isomers, and aminohydroxyphenylalanine (AHP) isomers, respectively.

to a HPLC system 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA). Optical rotation was measured using a Polarimeter (JASCO DIP-370; Tokyo, Japan).

Human NM pigments were isolated from SN and LC tissues as described previously (Wakamatsu *et al.* 2003, 2012; Zecca *et al.* 2008; Engelen *et al.* 2012). SN-NM was isolated from a pool of five subjects (average age 82 years old; 3 males and 2 females). LC-NM was isolated from a pool of 35 subjects (average age 77 years old; 22 males and 13 females). Brain samples were obtained during autopsies of male and female subjects who died without evidence of neuropsychiatric and neurodegenerative disorders. All tissues samples were analyzed anonymously. Samples of SN and LC were carefully dissected and then stored at –80°C until used for the isolation of NM pigment. In order to obtain comparable amounts of LC-NM and SN-NM, we had to pool LC tissues from many subjects since the LC brain area is much smaller than the SN. This study was approved by the Ethical Committee of the National Research Council of Italy—Institute of Biomedical Technologies (Segrate, Milan, Italy) and was carried out in agreement with the Policy of National Research Council of Italy.

HPLC conditions

For analysis of β-(carboxymethylthio)dopamine (CMT-DA) and other catecholamine metabolites formed upon HCl hydrolysis, the mobile phase was 0.1 M sodium citrate buffer, pH 3.0, containing 1 mM sodium octanesulfonate and 0.1 mM EDTA.2Na (buffer A): methanol, 95 : 5 (v/v) (Wakamatsu *et al.* 2003). Analyses were performed at 35°C with an electrochemical detector set at +700 mV versus Ag/AgCl electrode. For the analysis of 5-*S*-Cys-NE and 5-*S*-cysteiny β-(carboxymethylthio)dopamine (5-*S*-Cys-CMT-DA), we used a Capcell pak C₁₈ column with 0.1 M potassium phosphate buffer, pH 2.1: methanol, 99 : 1 (v/v), at 25°C, with a UV-VIS detector set at 290 nm. For the preparative separation of 5-*S*-Cys-NE, we used a Capcell pak C₁₈ column (type MG, 20 × 250 mm, 5 μm particle size; from Shiseido) attached with a Capcell pak C₁₈ column (20 × 35 mm, 5 μm particle size; from Shiseido), with 0.1 M potassium phosphate buffer, pH 2.1: methanol, 99 : 1 (v/v), at 55°C, respectively, with a UV-VIS detector set at 290 nm, at a flow

rate of 7.0 mL/min. Determination of melanin markers was performed using HPLC as previously reported (Wakamatsu *et al.* 2002, 2003; Ito *et al.* 2011).

HCl hydrolysis of human NM

One hundred μL of a suspension (1 mg/mL) of NM were transferred to a sealed-capped tube with 300 μL 6 M HCl containing 5% thioglycolic acid and 1% phenol. The tube was purged with a stream of argon, then sealed and heated at 110°C for 16 h. After cooling, the hydrolysate was mixed with 10 μL of an internal standard solution (methyl-5-*S*-Cys-DOPA, 15 $\mu\text{mol/L}$), extracted twice with 1 mL diethyl ether to remove phenol, and treated with alumina to extract catecholic compounds as follows. A 120 μL aliquot of the hydrolysate was transferred into an Eppendorf tube containing 50 mg acid-washed alumina and 200 μL 1% $\text{Na}_2\text{S}_2\text{O}_5$ -1% EDTA.2Na. To the mixture, 500 μL 2.7 M Tris.HCl-2% EDTA.2Na (pH 9.0) was added and immediately mixed vigorously for 5 min on a microtube mixer. After centrifugation, the aqueous layer was removed by aspiration, and the remaining alumina was washed with 1 mL water three times. The catecholic compounds were then eluted with 100 μL 0.4 M HClO_4 by shaking for 2 min. A 30 μL aliquot of the HClO_4 extract was injected into the HPLC system.

Synthesis of CMT-DA

A solution of 144 mg (0.7 mmol) NE.HCl in 20 mL 6 M HCl containing 5% thioglycolic acid and 1% phenol was refluxed for 30 min at 110°C. Thioglycolic acid and phenol were essential to avoid polymerization of the starting material NE. The reaction mixture was washed twice with 20 mL ether to remove phenol. The water layer was evaporated to dryness and then subjected to Dowex 50W-X2 chromatography (1.6 \times 10 cm in 2 M HCl). The column was eluted with 2 M HCl and fractions of 10 mL were collected. Fractions 9–16 were evaporated to give 159 mg (80% yield) of CMT-DA.HCl.H₂O salt as a colorless solid (HPLC purity: > 99%). UV λ_{max} 282 nm (ϵ 3,260) in 0.1 M HCl. ¹H-NMR (DCl): δ = 3.32 (dd, 2H, J = 16 Hz, 16 Hz), 3.47 (m, 2H), 4.19 (t, 1H, J = 8.0 Hz), 6.85 (dd, 1H, J = 2.3 Hz, J = 8.3 Hz), 6.93 (d, 1H, J = 8.3 Hz), 6.94 (d, 1H, J = 2.3 Hz). ¹³C-NMR (DCl): δ = 35.24, 45.53, 49.08, 118.26, 119.18, 123.47, 131.55, 147.17, 147.20, 176.79. Mass spectrum: m/z 244.0567 (M+H)⁺ for C₁₀H₁₄N₁O₄S₁ (−2.1 mDa). Elemental analysis: calculated for C₁₀H₁₃N₁O₄S₁.HCl.H₂O. C, 40.34%; H, 5.42%; N, 4.70%; S, 10.77%; Cl, 11.91%; found C, 40.72%; H, 5.27%; N, 4.72%; S, 10.79%; Cl, 13.05%.

Synthesis of 5-*S*-Cys-CMT-DA

A solution of 47 mg (0.16 mmol) 5-*S*-Cys-NE in 9.4 mL 6 M HCl containing 5% thioglycolic acid and 1% phenol was refluxed for 30 min at 110°C. The reaction mixture was washed twice with 20 mL ether to remove phenol. The water layer was evaporated to dryness and then subjected to Dowex 50W-X2 chromatography (1.6 \times 10 cm in 2 M HCl). The column was eluted with 2 M HCl and fractions of 10 mL were collected. Fractions 16–26 were evaporated to give 26 mg (14% yield) of 5-*S*-Cys-CMT-DA.HCl salt as a colorless solid (HPLC purity: 96%). UV λ_{max} 292 nm (ϵ 1,340) in 0.1 M HCl. ¹H-NMR (DCl): δ = 3.48 (m, 2H), 3.55 (m, 4H), 4.21 (m, 2H), 6.99 (d, 1H, J = 2.0 Hz), 7.10 (d, 1H, J = 2.4 Hz). ¹³C-NMR (DCl): δ = 35.2, 36.4, 45.4, 49.0, 54.7, 119.3, 121.0, 128.5, 131.7, 147.7, 148.6, 172.7, 176.7. ¹³C-NMR

shows that 5-*S*-Cys-CMT-DA consists of a mixture of diastereomers evidenced by a double series of signals. Mass spectrum: m/z 363.08 (M+H)⁺ for C₁₃H₁₈N₂O₆S₂.

Ion trap liquid chromatography/mass spectrometry of CMT-DA

Ion Trap Liquid Chromatography/Mass Spectrometry was performed using a liquid chromatography/mass spectrometry system by Esquire HCT Plus Mass Spectrometer (Bruker) connected to an HPLC system 1260 Infinity (Agilent Technologies) with a Cadenza CD-C₁₈ column (2.0 \times 75 mm) (Imtakt Corp., Kyoto, Japan) with 10% CH₃OH – 0.1% HCOOH, at flow rate of 0.1 mL/min.

Statistical analyses

We did not perform any statistical analyses, but values obtained by H₂O₂-mediated oxidation and HI hydrolysis of synthetic pheomelanins prepared from the Cys-derivatives of DA, NE, DOPAC, DOMA, DOPEG, DOPE, and DOPA, were obtained as a mean of two determinations.

Results

Alkaline H₂O₂-mediated oxidation and reductive HI hydrolysis

PDCA, PTCA, TDCA, and TTCA (Fig. 2) were measured using H₂O₂-mediated oxidation of synthetic pheomelanins prepared from Cys-DA, Cys-NE, Cys-DOPAC, Cys-DOPE, Cys-DOPA, Cys-DOMA, and Cys-DOPEG (Table 1). Pheomelanins prepared from Cys-DOPAC, Cys-DOPE, Cys-DOMA, and Cys-DOPEG did not give detectable levels of PTCA or PDCA, but produced only TDCA and TTCA, two specific markers of pheomelanin (Table 1). This is consistent with the fact that those Cys-catechols do not possess an amino group in their side chains.

On HI hydrolysis, DA-related pheomelanins, i.e., Cys-DA-melanin, Cys-DOPAC-melanin and Cys-DOPE-melanin, gave high levels of pheomelanin markers, i.e., 4-AHPEA, 4-AHPAA, and AHEB (as a mixture of 4-AHEB and 3-AHEB), respectively (Table 1 and Fig. 2). On the other hand, NE-related pheomelanins, i.e., Cys-NE-melanin, Cys-DOMA-melanin, and Cys-DOPEG-melanin, gave lower (a half to one-fifth of the corresponding DA-related pheomelanins) levels of those markers. Cys-DOPA-melanin gave a high level of 4-AHP.

Alkaline H₂O₂-mediated oxidation of SN-NM and LC-NM produced TDCA, TTCA, PDCA, and PTCA at levels similar between SN-NM and LC-NM. Levels of TDCA and TTCA were greater than those of PDCA and PTCA both in SN-NM and LC-NM, which is consistent with the incorporation of Cys as a benzothiazole moiety (Wakamatsu *et al.* 2012).

HI hydrolysis of SN-NM and LC-NM gave a similar pattern of catechols, i.e., DOPA, DA, and DOPAC and their aminohydroxyphenyl counterparts, i.e., 4-AHP, 4-AHPEA, and 4-AHPAA. AHEBs isomers were also detected (Table 1). DA was detected at the highest levels in both types of NMs, while NE was not detected (Wakamatsu *et al.*

Table 1 Chemical analysis of synthetic melanins prepared from Cys-DA, Cys-NE, Cys-DOPAC, Cys-DOPE, Cys-DOPA, Cys-DOMA and Cys-DOPEG, and human NMs isolated from SN and LC tissues

Melanin	PDCA nmol/mg	PTCA	TDCA	TTCA	4-AHPEA	4-AHPAA	AHEBs ^a	4-AHP	DA
Cys-DA ^b	13.6	0.60	25.8	62.5	644	ND ^c	23.7	ND	20.5
Cys-NE ^b	4.39	0.40	16.2	77.9	326	ND	11.7	ND	ND
Cys-DOPAC ^b	ND	ND	18.6	40.8	ND	1256	ND	ND	ND
Cys-DOPE ^b	ND	ND	32.8	37.8	ND	ND	866	ND	ND
Cys-DOPA ^b	5.67	15.3	12.0	34.1	ND	ND	ND	539	ND
Cys-DOMA ^b	ND	ND	24.5	67.4	ND	537	ND	ND	ND
Cys-DOPEG ^b	ND	ND	21.4	68.9	ND	ND	151	ND	ND
SN-NM ^d	1.3	0.27	3.5	4.1	2.8 ^e	4.4 ^e	0.24 ^e	0.32 ^e	8.0 ^e
LC-NM ^d	2.6	0.34	3.8	6.4	5.3 ^e	3.6 ^e	0.55 ^e	0.26 ^e	6.2 ^e

^aAHEBs were estimated as a mixture of 4-AHEB and 3-AHEB.

^bValue obtained as a mean of two determinations.

^cNot detected.

^dValue obtained in a single determination.

^eData from Wakamatsu *et al.* 2014.

2014). In this study, we did not detect 6-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole or 7-(2-amino-2-carboxyethyl)-2-carboxy-4-hydroxybenzothiazole in alkaline H₂O₂-mediated oxidation mixtures known as any benzothiazole markers (Greco *et al.* 2009). These results suggest two alternative possibilities: (i) that SN-NM and LC-NM have common structural features characterized by the incorporation of DOPA, DA, and DOPAC, or (ii) that LC-NM is produced, at least in part, from NE (and DOPEG and DOMA), but the resulting structural features cannot be confirmed because of the reduction in the benzylic hydroxyl group to the hydrogen atom in NE (and DOPEG and DOMA).

Syntheses of CMT-DA and 5-S-Cys-CMT-DA

HCl hydrolysis of NM pigments is one approach to characterize the structure of NM. A high level of DA was previously detected in an HCl hydrolysate of SN-NM (Zecca *et al.* 2008). In order to establish a method to detect NE or NE-derived products, we performed HCl hydrolysis of NE itself. Hydrolysis with 6 M HCl containing 5% thioglycolic acid and 1% phenol was carried out using the method of protein hydrolysis at 110°C for 16 h (Murakami *et al.* 2007; Zecca *et al.* 2008). Phenol is usually added when proteins are hydrolyzed with HCl. Phenol plays a role in protecting phenolic and catecholic compounds from oxidation (Gieseg *et al.* 1993). As expected, NE (with a retention time of 4.7 min) disappeared from the HPLC chromatogram, and instead an unknown compound newly appeared at the retention time of 16.1 min. In the absence of thioglycolic acid, the reaction resulted in the production of a black polymeric material. The new compound was prepared in a large scale and purified by ion-exchange chromatography in

a 81% yield. We performed instrumental analyses, including ¹H-NMR, ¹³C-NMR, High Resolution Mass Spectrometry (HRMS) spectra, Ion Trap Liquid Chromatography/Mass Spectrometry, and elemental analysis, to determine the structure of this compound. ¹H-NMR and ¹³C-NMR spectra indicated the existence of one benzyl proton (in addition to other protons), three aliphatic carbons, six aromatic carbons, and one carbonyl carbon. The elemental analysis of the compound confirmed the existence of sulfur and chlorine atoms in a ratio of 1 : 1, and HRMS monitored by mass spectrometry in positive mode showed a 244 *m/z* ion peak for a 243-dalton compound (see Supporting Information). Extracted-ion chromatogram (EIC) in Ion Trap Liquid Chromatography/Mass Spectrometry of CMT-DA appeared as a broad peak at 4.5 min, which may contain a mixture of enantiomers of CMT-DA because this compound is racemic, as proved by its optical rotation ($[\alpha]_D = 0^\circ$). Thus, we identified the new unknown compound as CMT-DA. On the other hand, HCl hydrolysis of DA under the same conditions did not afford CMT-DA.

We also synthesized 5-S-Cys-CMT-DA in a 14% yield by HCl hydrolysis of 5-S-Cys-NE in the presence of thioglycolic acid and phenol. The structure of 5-S-Cys-CMT-DA was identified as the 5-S-Cys derivative of CMT-DA by several instrumental analyses, including ¹H-NMR, ¹³C-NMR and HRMS (see Supporting Information). HRMS monitored by mass spectrometry in positive mode showed a 363 *m/z* ion peak for a 362-dalton compound, 5-S-Cys-CMT-DA (see Supporting Information).

HCl hydrolysis of SN-NM and LC-NM

HCl hydrolysis of SN-NM in the presence of thioglycolic acid and phenol gave DA (31.4 nmol/mg) and DOPA

(19.7 nmol/mg) as major products, in addition to DOPAC (5.4 nmol/mg), Cys-DA (1.7 nmol/mg), Cys-DOPA (0.9 nmol/mg), and Cys-DOPAC (1.4 nmol/mg) (Figs 3a and 4a). CMT-DA and 5-*S*-Cys-CMT-DA were not detected on the HPLC chromatogram (Fig. 3a).

HCl hydrolysis of human LC-NM in the presence of thioglycolic acid and phenol produced CMT-DA (92.7 nmol/mg) as an exceedingly major product, in addition to DA (18.4 nmol/mg), DOPA (8.5 nmol/mg) and DOPAC (2.7 nmol/mg). A low level of 5-*S*-Cys-CMT-DA (2.7 nmol/mg) was also detected. Cys-DOPA was detected in a low amount (1.1 nmol/mg), while Cys-DA and Cys-DOPAC were below the detection limits (Figs 3b and 4b). CMT-DA and 5-*S*-Cys-CMT-DA were identified by co-injection with authentic samples. The Ion Trap Liquid Chromatography/Mass Spectrometry of the HCl hydrolysate of LC-NM showed two EICs which were 5-*S*-Cys-CMT-DA at 3.8–4.0 min and CMT-DA at 4.4 min. 5-*S*-Cys-CMT-DA at 3.8–4.0 min and CMT-DA at 4.4 min in EIC appeared at the separated and broad peaks, respectively. The reason why EIC of 5-*S*-Cys-CMT-DA showed two peaks may be explained by ^{13}C -NMR that 5-*S*-Cys-CMT-DA consists of a mixture of diastereomers evidenced by a double series of signals. (see Supporting Information). The above results indicate that in addition to smaller amounts of DA and DOPA as biosynthetic precursors of NE, a greater amount of NE (and 5-*S*-Cys-NE) is incorporated into LC-NM.

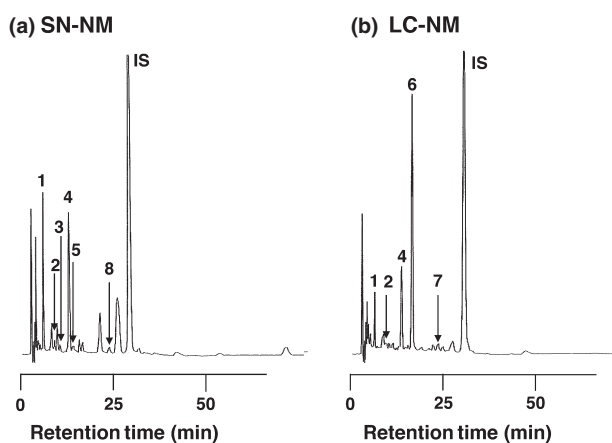


Fig. 3 HPLC chromatograms of HCl hydrolysates of NM from SN tissue (SN-NM) and NM from LC tissue (LC-NM). Hydrolysis of SN-NM (a) and LC-NM (b) by 6 M HCl was performed in the presence of 5% thioglycolic acid and 1% phenol for 16 h at 110°C. The hydrolysates were treated with alumina to extract catecholic compounds in the presence of the IS (internal standard) α -methyl-Cys-DOPA (Wakamatsu and Ito 1994). #1: 3,4-dihydroxyphenylalanine (DOPA), #2: 3,4-dihydroxyphenylacetic acid (DOPAC), #3: 5-*S*-Cys-DOPA, #4: dopamine (DA), #5: 5-*S*-Cys-DOPAC, #6: β -(carboxymethylthio)dopamine (CMT-DA), #7: 5-*S*-cysteinyl β -(carboxymethylthio)dopamine (5-*S*-Cys-CMT-DA), #8: 5-*S*-Cys-DA. IS is methyl Cys-DOPA as an internal standard.

Discussion

NM mainly accumulates in catecholaminergic neurons of the SN and LC (Zecca *et al.* 2004) and its structure differs from peripheral melanins produced in the hair, skin, and eyes (Bogerts 1981; Saper and Petito 1982; Zecca *et al.* 2003).

In contrast to the rather extensive studies on the biosynthetic origin and structure of SN-NM (Wakamatsu *et al.* 2003, 2012; Zecca *et al.* 2008), the chemical constituents of LC-NM have not been studied in detail as far as we know. In particular, whether NE is actually incorporated in LC-NM is not known. In this study, the H_2O_2 -mediated oxidation and HI hydrolysis of SN-NM and LC-NM failed to clearly differentiate LC-NM from SN-NM but confirmed their similarities. In continuous efforts to find a new clue for the incorporation of NE into LC-NM, we performed HCl hydrolysis of SN-NM and LC-NM. Our previous study (Zecca *et al.* 2008) showed that approximately 90% of catechols, DOPA, DA, Cys-DOPA, and Cys-DA, are present in the protein-bound form. Therefore, in this study, we did not attempt to separate catechols into the free and protein-bound forms. The HCl hydrolysis of SN-NM yielded DOPA, DA, and DOPAC, together with Cys-DOPA, Cys-DA, and Cys-DOPAC (Figs 3a and 4a). On the other hand, the HCl hydrolysis of LC-NM gave a high level of an unknown catechol, in addition to DOPA, DA, and DOPAC (Figs 3b and 4b). This compound was then identified as CMT-DA, produced *via* the substitution of the hydroxyl group of NE with the carboxymethylthio group of thioglycolic acid. Thus, we propose a mechanism (see Supporting Information) for the production of CMT-DA showing that an addition reaction of thioglycolic acid may occur at the benzyl position of NE via the quinonemethide produced from the elimination of a hydroxyl group at the benzyl position. HCl hydrolysis of DA under the same conditions did not afford CMT-DA. This showed that the production of CMT-DA does not proceed through oxidation of DA to the quinonemethide, but the elimination of a hydroxyl group at the benzyl position of NE. Another possible mechanism may be via a carbonium ion at the benzyl position after elimination of the hydroxyl group. These mechanisms are supported by the experimental result that this compound is racemic, as proved by its optical rotation ($[\alpha]_{\text{D}} = 0^\circ$). 5-*S*-Cys-DMT-DA, arising from 5-*S*-Cys-NE, was also detected in a small amount. Although the exact nature of NE binding to LC-NM proteins (and/or pigment) was not clarified in this study, one possibility may be that NE is trapped by proteins/pigment *via* Schiff base formation (nitrogen-carbon double bond).

This study also suggests that DA and its metabolites, DOPAC and DOPE, are responsible for the production of SN-NM, while NE and its metabolites, DOMA and DOPEG, are responsible for the production of LC-NM. In reductive HI hydrolysis of LC-NM, comparable amounts of DA and 4-AHPEA were obtained, as previously reported (Wakamatsu

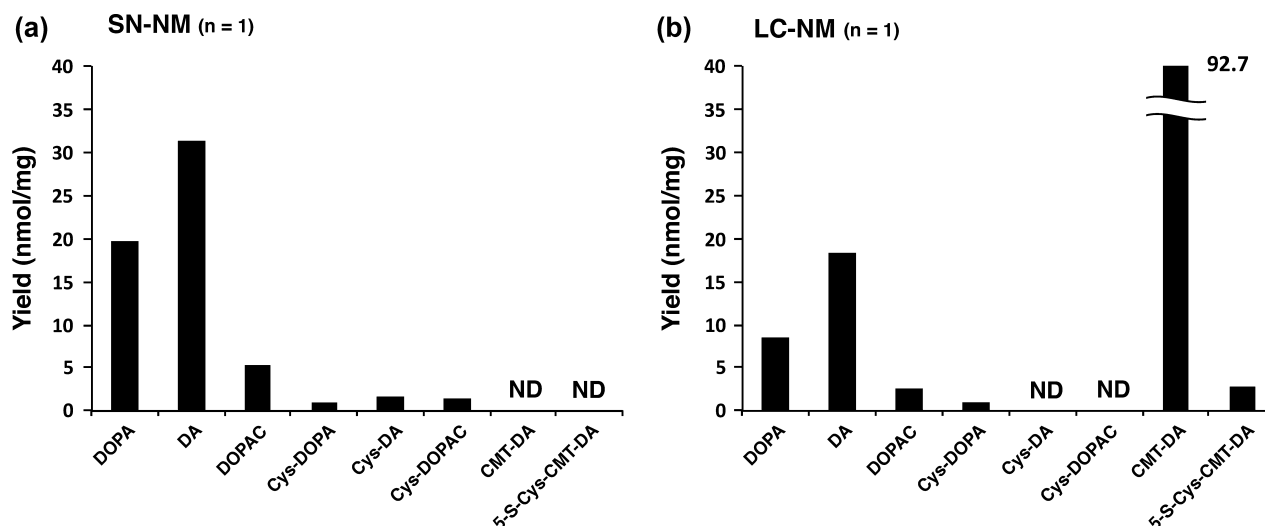


Fig. 4 Hydrolysis of NM from SN tissue (SN-NM) (a) and NM from LC tissue (LC-NM) (b) by 6 M HCl in the presence of thioglycolic acid. ND, not detected.

et al. 2014). This suggests that they may be produced by the elimination of hydroxyl groups from NE and the corresponding aminohydroxy derivative having a hydroxyl group at the benzyl position of 4-AHPEA (derived from Cys-NE-melanin). Moreover, DOPA and DA, present in LC neurons as precursors of NE synthesis, are incorporated in LC-NM.

NM pigment is formed by the oxidative polymerization of catecholamines in SN neurons (Mann and Yates 1974; Graham 1979; Bogerts 1981; Sulzer *et al.* 2000; Napolitano *et al.* 2011). It has been reported that NM is a compound containing a melanic component (eumelanin dihydroxyindole and pheomelanin benzothiazine/benzothiazole units) bound to polyisoprenic and proteic components (Zecca *et al.* 2000, 2008; Engelen *et al.* 2012; Wakamatsu *et al.* 2012). It has been demonstrated in brain tissue that DA-quinone synthesized by the non-enzymatic autoxidation of DA (Zhang and Dryhurst 1993) reacts with the thiol group of L-Cys/ glutathione forming 5-S-Cys-DA/5-S-glutathionyl-DA, the latter being subsequently hydrolyzed by peptidases to produce 5-S-Cys-DA (Napolitano *et al.* 2011; Segura-Aguilar *et al.* 2014). The presence of 5-S-Cys-DA in the human SN and in other brain regions rich in dopamine (DA) has been described, suggesting the possible incorporation of 5-S-Cys-DA into NM pigment (Rosengren *et al.* 1985).

DA-quinone was reported to cyclize several-fold more slowly to produce the corresponding aminochrome than does DOPA-quinone. This makes DA-quinone highly reactive and toxic (Land and Riley 2000; Segura-Aguilar *et al.* 2001; Ito and Wakamatsu 2008). Thus, DA may exert its toxicity through oxidation to DA-quinone followed by binding to SH enzymes that are essential for cell proliferation and/or survival. Interestingly, levels of Cys-DA are known to be elevated in the brains of patients who died from PD (Vauzour *et al.* 2008). The toxicity results from the facile autoxidation

of these intermediates, thus producing reactive oxygen species. PD selectively targets catecholaminergic neurons, with a variable degree of involvement of the LC compared to the SN (Paulus and Jellinger 1991; Gesi *et al.* 2000; Zarow *et al.* 2003; Cebrián *et al.* 2014). It was clearly shown that patients with PD show a severe decrease of NM levels in their SN (around 60% compared to age-matched controls) due to the loss of neurons containing NM (Zecca *et al.* 2002).

On the other hand, the noradrenergic neurotransmitter NE is also very easily oxidized at physiological pH to an *o*-quinone that normally cyclizes and subsequently oxidatively polymerizes to black melanin. However, the oxidative behavior of NE is not much different from that of DA because the chemical reactivity is primarily dictated by the catechol moiety (Napolitano *et al.* 2011). As a matter of fact, besides the enzymatic oxidative deamination to DOMA and H₂O₂ and the subsequent degradation to various metabolites, NE also may take part in the formation of NM *via* a process that involves oxidation to NE-*o*-quinone followed by intramolecular cyclization. These reactive NE-*o*-quinones can also react with Cys or glutathione to produce neurotoxic compounds (Xin *et al.* 2000; Shen and Dryhurst 2001). For example, it is likely that NE-*o*-quinone could be scavenged by Cys leading to 5-S-Cys-NE by the same mechanism as known for DA, thus influencing the vulnerability of noradrenergic neurons. An alternative (protective) pathway could be the following oxidation which may contribute to NM formation *via* a complex polymerization process (Manini *et al.* 2007). As previously described for NM biosynthesis in dopaminergic SN neurons (Sulzer *et al.* 2000), the synthesis of NM inside LC neurons could also be a protective process which removes these NE-derived toxic compounds.

In conclusion, we demonstrated in this study that NE and 5-S-Cys-NE are incorporated into LC-NM by identifying two new compounds, CMT-DA and 5-S-Cys-CMT-DA. We found that SN-NM and LC-NM contain melanin derived not only from DA and NE but also from several other catecholic metabolites that occur in catecholaminergic neurons such as DOPAC, DOPE, DOPA, DOMA, and DOPEG, in addition to the corresponding Cys-derivatives in varying degrees. It is reasonable to conclude that DA, DOPAC, DOPE, and DOPA are mainly responsible for the structure of SN-NM, while NE, DOMA, and DOPEG are responsible for the structure of LC-NM in addition to DA, DOPAC, DOPE, and DOPA since abundant amounts of DA occur in LC neurons as a precursor of NE synthesis.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. ¹H-NMR spectra of CMT-DA.

Figure S2. ¹³C-NMR spectra of CMT-DA.

Figure S3. HRMS spectra of CMT-DA.

Figure S4. ¹H-NMR spectra of 5-S-Cys-CMT-DA.

Figure S5. ¹³C-NMR spectra of 5-S-Cys-CMT-DA.

Figure S6. HRMS spectra of 5-S-Cys-CMT-DA.

Figure S7. Extracted-ion chromatogram (EIC) in Ion Trap LC-MS of CMT-DA.

Figure S8. Extracted-ion chromatogram (EIC) in Ion Trap LC-MS of LC-NM.

Figure S9. Proposed mechanism for the production of CMT-DA by hydrolysis of NE with 6 M HCl in the presence of thioglycolic acid and phenol.

References

Bogerts B. (1981) A brainstem atlas of catecholaminergic neurons in man, using melanin as natural marker. *J. Comp. Neurol.* **197**, 63–80.

Cebrián C., Zucca F. A., Mauri P. *et al.* (2014) MHC-I expression renders catecholaminergic neurons susceptible to T-cell-mediated degeneration. *Nat. Commun.* **5**, 3633.

Engelen M., Vanna R., Bellei C., Zucca F. A., Wakamatsu K., Monzani E., Ito S., Casella L. and Zecca L. (2012) Neuromelanins of human brain have soluble and insoluble components with dolichols attached to the melanic structure. *PLoS ONE* **7**, e48490.

Gesi M., Soldani P., Giorgi F. S., Santinami A., Bonaccorsi I. and Fornai F. (2000) The role of the locus coeruleus in the development of Parkinson's disease. *Neurosci. Biobehav. Rev.* **24**, 655–668.

Gieseg S. P., Simpson J. A., Charlton T. S., Duncan M. W. and Dean R. T. (1993) Protein-bound 3,4-dihydroxyphenylalanine is a major reductant formed during hydroxyl radical damage to proteins. *Biochemistry* **32**, 4780–4786.

Graham D. G. (1979) On the origin and significance of neuromelanin. *Arch. Pathol. Lab. Med.* **103**, 359–362.

Greco G., Wakamatsu K., Panzella L., Ito S., Napolitano A. and d'Ischia M. (2009) Isomeric cysteinyl dopas provide a (photo) degradable bulk component and a robust structural element in red human hair pheomelanin. *Pigment Cell Melanoma Res.* **22**, 319–327.

d'Ischia M., Wakamatsu K., Napolitano A. *et al.* (2013) Melanins and melanogenesis: methods, standards, protocols. *Pigment Cell Melanoma Res.* **26**, 616–633.

Ito S. (1989) Optimization of conditions for preparing synthetic pheomelanin. *Pigment Cell Res.* **2**, 53–56.

Ito S. and Fujita K. (1985) Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. *Anal. Biochem.* **144**, 527–536.

Ito S. and Wakamatsu K. (2003) Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. *Pigment Cell Res.* **16**, 523–531.

Ito S. and Wakamatsu K. (2008) Chemistry of mixed melanogenesis: pivotal roles of dopaquinone. *Photochem. Photobiol.* **84**, 582–592.

Ito S., Fujita K., Yoshioka M., Sienko D. and Nagatsu T. (1986) Identification of 5-S- and 2-S-cysteinyl dopamine and 5-S-glutathionyl dopamine formed from dopamine by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.* **375**, 134–140.

Ito S., Nakanishi Y., Valenzuela R. K., Brilliant M. H., Kolbe L. and Wakamatsu K. (2011) Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins. *Pigment Cell Melanoma Res.* **24**, 605–613.

Land E. J. and Riley P. A. (2000) Spontaneous redox reactions of dopaquinone and the balance between the eumelanin and pheomelanin pathways. *Pigment Cell Res.* **13**, 273–277.

Manini P., Panzella L., Napolitano A. and d'Ischia M. (2007) Oxidation chemistry of norepinephrine: partitioning of the O-quinone between competing cyclization and chain breakdown pathways and their roles in melanin formation. *Chem. Res. Toxicol.* **20**, 1549–1555.

Mann D. M. A. and Yates P. O. (1974) Lipoprotein pigments- their relationship to ageing in the human nervous system. II. The melanin content of pigmented nerve cells. *Brain* **97**, 489–498.

Mann D. M. A. and Yates P. O. (1983) Possible role of neuromelanin in the pathogenesis of Parkinson's disease. *Mech. Ageing Dev.* **21**, 193–203.

Marsden C. D. (1983) Neuromelanin and Parkinson's disease. *J. Neural Transm. Suppl.* **19**, 121–141.

Murakami K., Wakamatsu K., Nakanishi Y., Takahashi H., Sugiyama S. and Ito S. (2007) Serum levels of pigmentation markers are elevated in patients undergoing hemodialysis. *Blood Purif.* **25**, 483–489.

Napolitano A., Manini P. and d'Ischia M. (2011) Oxidation chemistry of catecholamines and neuronal degeneration: an update. *Curr. Med. Chem.* **18**, 1832–1845.

Paulus W. and Jellinger K. (1991) The neuropathologic basis of different clinical subgroups of Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **50**, 743–755.

- Rosengren E., Linder-Eliasson E. and Carlsson A. (1985) Detection of 5-S-cysteinyl-dopamine in human brain. *J. Neural. Transm.* **63**, 247–253.
- Saper C. B. and Petito C. K. (1982) Correspondence of melanin-pigmented neurons in human brain with A1-A14 catecholamine cell groups. *Brain* **105**, 87–101.
- Segura-Aguilar J., Metodiewa D. and Baez S. (2001) The possible role of one-electron reduction of aminochrome in the neurodegenerative process of the dopaminergic system. *Neurotox. Res.* **3**, 157–165.
- Segura-Aguilar J., Paris I., Muñoz P., Ferrari E., Zecca L. and Zucca F. A. (2014) Protective and toxic roles of dopamine in Parkinson's disease. *J. Neurochem.* **129**, 898–915.
- Shen X. M. and Dryhurst G. (2001) Influence of glutathione on the oxidation chemistry of 5-S-cysteinyl-dopamine: potentially neuroprotective reactions of relevance to Parkinson's disease. *Tetrahedron* **57**, 393–405.
- Sulzer D., Bogulavsky J., Larsen K. E. *et al.* (2000) Neuromelanin biosynthesis is driven by excess cytosolic catecholamines not accumulated by synaptic vesicles. *Proc. Natl Acad. Sci. USA* **97**, 11869–11874.
- Sulzer D., Mosharov E., Talloczy Z., Zucca F. A., Simon J. D. and Zecca L. (2008) Neuronal pigmented autophagic vacuoles: lipofuscin, neuromelanin, and ceroid as macroautophagic responses during aging and disease. *J. Neurochem.* **106**, 24–36.
- Vauzour D., Ravaioli G., Vafeiadou K., Rodriguez-Mateos A., Angeloni C. and Spencer J. P. E. (2008) Peroxynitrite induced formation of the neurotoxins 5-S-cysteinyl-dopamine and DHBT-1: implications for Parkinson's disease and protection by polyphenols. *Arch. Biochem. Biophys.* **476**, 145–151.
- Wakamatsu K. and Ito S. (1994) Improved HPLC determination of 5-S-cysteinyl-dopa in serum. *Clin. Chem.* **40**, 495–496.
- Wakamatsu K., Ito S. and Rees J. L. (2002) Usefulness of 4-amino-3-hydroxyphenylalanine as a specific marker of pheomelanin. *Pigment Cell Res.* **15**, 225–232.
- Wakamatsu K., Fujikawa K., Zucca F. A., Zecca L. and Ito S. (2003) The structure of neuromelanin as studied by chemical degradative methods. *J. Neurochem.* **86**, 1015–1023.
- Wakamatsu K., Murase Y., Zucca F. A., Zecca L. and Ito S. (2012) Biosynthetic pathway to neuromelanin and its aging process. *Pigment Cell Melanoma Res.* **25**, 793–802.
- Wakamatsu K., Tanaka H., Tabuchi K., Ojika M., Zucca F. A., Zecca L. and Ito S. (2014) Reduction of the nitro group to amine by hydroiodic acid to synthesize *o*-aminophenol derivatives as putative degradative markers of neuromelanin. *Molecules* **19**, 8039–8050.
- Xin W., Shen X. M., Li H. and Dryhurst G. (2000) Oxidative metabolites of 5-S-cysteinyl-norepinephrine are irreversible inhibitors of mitochondrial complex I and the alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes: possible implications for neurodegenerative brain disorders. *Chem. Res. Toxicol.* **13**, 749–760.
- Zarow C., Lyness S. A., Mortimer J. A. and Chui H. C. (2003) Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. *Arch. Neurol.* **60**, 337–341.
- Zecca L., Costi P., Mecacci C., Ito S., Terreni M. and Sonnino S. (2000) Interaction of human substantia nigra neuromelanin with lipids and peptides. *J. Neurochem.* **74**, 1758–1765.
- Zecca L., Fariello R., Riederer P., Sulzer D., Gatti A. and Tampellini D. (2002) The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease. *FEBS Lett.* **510**, 216–220.
- Zecca L., Zucca F. A., Wilms H. and Sulzer D. (2003) Neuromelanin of the substantia nigra: a neuronal black hole with protective and toxic characteristics. *Trends Neurosci.* **26**, 578–580.
- Zecca L., Stroppolo A., Gatti A. *et al.* (2004) The role of iron and copper molecules in the neuronal vulnerability of locus coeruleus and substantia nigra during aging. *Proc. Natl Acad. Sci. USA* **101**, 9843–9848.
- Zecca L., Zucca F. A., Albertini A., Rizzio E. and Fariello R. G. (2006) A proposed dual role of neuromelanin in the pathogenesis of Parkinson's disease. *Neurology* **67**(7 Suppl 2), S8–S11.
- Zecca L., Bellei C., Costi P. *et al.* (2008) New melanic pigments in the human brain that accumulate in aging and block environmental toxic metals. *Proc. Natl Acad. Sci. USA* **105**, 17567–17572.
- Zhang F. and Dryhurst G. (1993) Oxidation chemistry of dopamine: possible insights into the age-dependent loss of dopaminergic nigrostriatal neurons. *Bioorg. Chem.* **21**, 392–410.